JOURNAL OF VIROLOGY, Aug. 2001, p. 7193–7197 Vol. 75, No. 15
0022-538X/01/$04.00+0 DOI: 10.1128/JVI.75.15.7193–7197.2001

Context-Dependent Phenotype of a Human Immunodeficiency Virus Type 1 Nucleocapsid Mutation

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Received 31 October 2000/Accepted 3 May 2001

The human immunodeficiency virus type 1 (HIV-1) nucleocapsid mutation R10A/K11A abolishes viral replication when present in proviral clone HIV-1_{HXB-2}, but it was found to have minimal effect on replication of the closely related HIV-1_{NL4-3}. Functional mapping demonstrated that a nonconservative amino acid change at nucleocapsid residue 24 (threonine in HIV-1_{HXB-2}, isoleucine in HIV-1_{NL4-3}) is the major determinant of the different R10A/K11A phenotypes in these two proviruses. Threonine-isoleucine exchanges appear to modify the R10A/K11A phenotype via effects on virion RNA-packaging efficiency. The improved packaging seen with hydrophobic isoleucine is consistent with solution structures localizing this residue to a hydrophobic pocket that contacts guanosine bases in viral genomic RNA stem-loops critical for packaging.

Retroviral nucleocapsid (NC) carries out important functions throughout the entire retroviral life cycle (2, 19). Upon translation as part of the Gag polyprotein, NC mediates Gag multimerization and virion assembly and directs the packaging of two copies of viral genomic RNA into virions. After Gag polyprotein processing by the virus-encoded protease, NC coats viral genomic RNA and subsequently influences early events in the viral life cycle such as reverse transcription and possibly even integration (3, 4, 8, 12). Each of these functions seems to require RNA binding on the part of NC. Genetic and structural studies indicate that conserved Cys-His boxes of NC mediate specific viral genomic RNA packaging by pairing with *cis*-acting stem-loops on the RNA (1, 9, 14, 18). The specificity of RNA binding seems less important for other NC functions, such as virion assembly and reverse transcription; here, NC basic residues mediate nonspecific binding of NC to RNA via electrostatic interactions with the phosphodiester groups of the RNA (1, 7, 9).

Context-dependent replication of the R10A/K11A mutation. To elucidate the function of human immunodeficiency virus type 1 (HIV-1) NC basic residues, we and others previously characterized a panel of alanine-scanning mutations (7, 17). Among these mutations, R10A/K11A was introduced into the HIV-1_{HXB-2} provirus and found to disrupt viral replication. This mutation substitutes alanine at positions that are invariably basic among different HIV-1 isolates (15). We therefore expected to observe similar negative effects on viral replication when we introduced this mutation into other proviral clones.

Using standard techniques (20), R10A/K11A was introduced into HIV-1_{NL4-3}, a proviral clone closely related to HIV-1_{HXB-2}. Replication studies were performed as previously described (7): virions produced by transfection of proviral DNAs into 293T cells were normalized by exogenous reverse transcriptase (RT) activity and used to initiate infection of Jurkat T cells (22). Every 2 days cells were passaged and supernatant was collected. Evidence for virus spread through the culture was obtained by measuring exogenous RT activity in the culture supernatant. Levels of wild-type HIV-1_{NL4-3} peaked about 8 days postinfection (Fig. 1). Levels of wild-type HIV-1_{HXB-2} peaked slightly later, an observation consistent with the fact that this clone has defects in three accessory genes: *vpu, nef* and *vpr* (10, 11). As previously reported, replication of the HIV-1_{HXB-2} R10A/K11A mutant was abolished (17) and no exogenous RT activity above the background could be detected (Fig. 1). Much to our surprise, when the R10A/K11A mutation was introduced into proviral clone HIV-1_{NL4-3}, the virus was able to replicate robustly (Fig. 1), albeit with slower kinetics than wild-type HIV-1_{NL4-3}.

Mapping of the difference between HIV-1_{NL4-3} and HIV-1_{HXB-2} that determines the phenotype of the R10A/K11A mutation. The striking difference in replication between HIV-1_{NL4-3} and HIV-1_{HXB-2} clones harboring the R10A/K11A mutation could not be explained by differences in accessory genes or other sequences 3′ of *pol*. First, exchanging these sequences between HIV-1_{NL4-3} and HIV-1_{HXB-2} did not modify the R10A/K11A phenotype (data not shown). Second, we have previously shown that transfer into HIV-1_{NL4-3} of a *Spe*I-*Eco*RV fragment from HIV-1_{HXB-2} (nucleotides 1507 to 2977 from the middle of CA to the middle of RT) is sufficient to render R10A/K11A unable to replicate (8). This indicates that the determinant of the R10A/K11A phenotype lies within the *Spe*I-*Eco*RV fragment (Fig. 2a).

To restrict the determinant of the R10A/K11A phenotype further, a second chimeric virus was engineered in which a *Spe*I-*Apa*I fragment from HIV-1_{HXB-2} (nucleotides 1507 to 2006) was substituted for corresponding sequences in HIV-1_{NL4-3} (Fig. 2a). Virions were used to infect Jurkat T cells, and infections were analyzed as above. Replication of either "wild-type" chimeric virus, NL4-3/$HX_{(1507–2977)}$ or NL4-3/$HX_{(1507–2006)}$, was similar to that of wild-type HIV-1_{NL4-3} (Fig. 2b), as previously described (8). In contrast, when the R10A/K11A mu-

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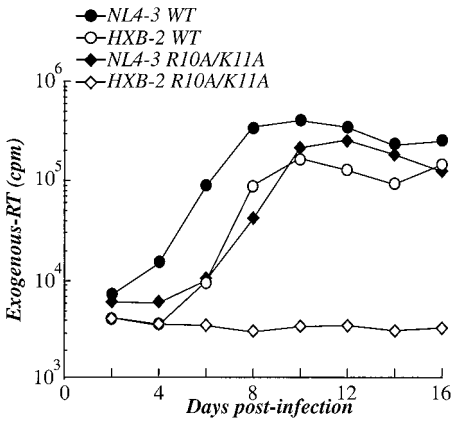


FIG. 1. Replication of wild-type (WT) and R10A/K11A mutant viruses in the HIV-1_{NL4-3} and HIV-1_{HXB-2} proviral backgrounds. Jurkat T cells were infected with virus stocks (as indicated) produced by transient transfection and normalized by exogenous RT activity. After infection, cells were passaged every 2 days and supernatant was collected. The accumulation of exogenous RT activity in the supernatant of infected cells (ordinate) is shown for the indicated day postinfection (abscissa).

tation was present in either of these two chimeric viruses, replication was severely impaired, as it is when the mutation is present in HIV-1_{HXB-2} (Fig. 2b). These results indicate that the determinant for the R10A/K11A replication phenotype is within sequences encoding the C terminus of CA through the first zinc finger of NC. When HIV-1_{NL4-3} and HIV-1_{HXB-2} sequences from this region were compared, eight amino acid differences were found: two in CA, one in SPI, and five in NC. Three of the latter are conservative changes, while two are nonconservative changes (Fig. 3a).

To determine if any of these nonconserved amino acids is sufficient to determine the phenotype of the R10A/K11A mutation, we changed HIV-1_{HXB-2} residues to their HIV-1_{NL4-3} counterparts and asked if these would rescue replication of the HIV-$1_{NL4-3/HXB-2}$ R10A/K11A chimera. We focused our attention on the two nonconservative amino acid changes present at positions 12 and 24 of NC (Fig. 3a). Individual substitutions in NC were engineered by PCR as previously described (6), and the amplified products, digested with the restriction enzymes *Spe*I and *Apa*I, were used to replace the corresponding fragment of the specific proviral clone. Jurkat T cells were then infected with mutant viruses, and viral replication was examined (Fig. 3b). Changing the isoleucine residue encoded by HIV-1_{HXB-2} at NC position 12 to the threonine encoded by HIV-1_{NL4-3} failed to rescue replication of NL4-3/$HX_{(1507–2977)}$ R10A/K11A (Fig. 3b). In contrast, NL4-3/$HX_{(1507–2977)}$ R10A/K11A/T24I replicated quite well (Fig. 3b), suggesting that NC residue 24 could be the major determinant of the R10A/K11A phenotype.

The experiments described above tested the importance of NC residue 24 in the context of HIV-$1_{NL4-3/HXB-2}$ chimeric viruses. To formally demonstrate that residue 24 is responsible for the context-dependent phenotype of the R10A/K11A mutation, residue 24 substitutions were introduced into nonchimeric HIV-1 proviruses. Change of the T24 residue to an isoleucine was sufficient to rescue replication of the HIV-1_{HXB-2} R10A/K11A mutant to a level similar to that of wild-type HIV-1_{HXB-2} (Fig. 3c). In addition, the reciprocal change of I24 to threonine impaired the replication of HIV-1_{NL4-3} R10A/K11A (Fig. 3d). None of the solo changes at position 24 in the absence of other mutations affected viral replication (Fig. 3c and d). These results formally demonstrate that the difference between NC residue 24 in HIV-1_{HXB-2} and HIV-1_{NL4-3} is the primary determinant of the R10A/K11A phenotype. However, since exogenous RT activity clearly distinguishable from the background accumulated in cultures infected with HIV-1_{NL4-3} R10A/K11A/I24T, these data show that other primary sequence differences between the two viral clones must contribute to the replication of the HIV-1_{NL4-3} R10A/K11A mutant.

Characterization of mutant virions. The effect on the phenotype of retroviruses harboring the R10A/K11A mutation of changing the identity of NC residue 24 between isoleucine and threonine was examined next in a single-cycle replication assay. Virions produced by transfection of 293T cells were purified by ultracentrifugation through 25% sucrose, normalized by exogenous RT activity, and then used to infect $CD4^+$ HeLa cells containing a β-galactosidase reporter (13). The infectivity of the virion preparations was then quantitated as previously described (8). Consistent with the delayed kinetics shown in Fig. 1, wild-type HIV-1_{HXB-2} virions were found to be two- to

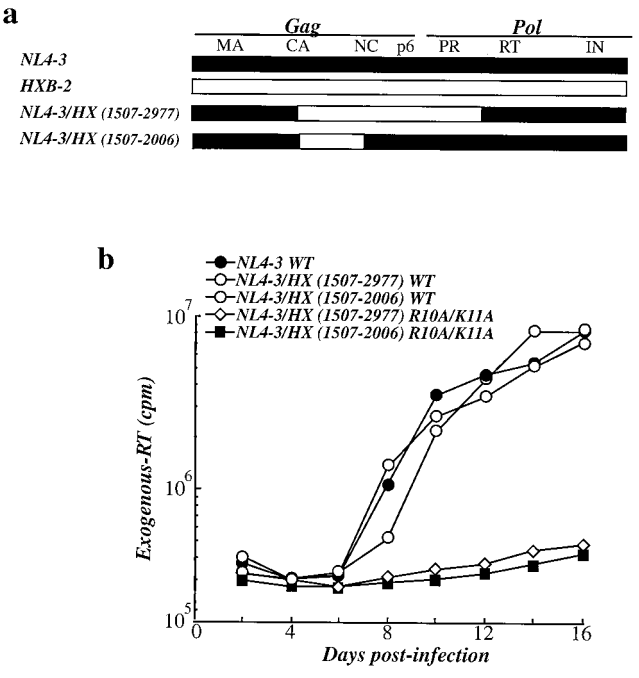


FIG. 2. Effect of the R10A/K11A mutation on replication of HIV-$1_{NL4-3/HXB-2}$ chimeric viruses, indicated here as NL4-3/HX. (a) Schematic representation of *gag* and *pol* in the virus chimeras used here. Sequences from HIV-1_{NL4-3} or HIV-1_{HXB-1} are represented by black and white bars, respectively. The proviral nucleotide numbers flanking the regions exchanged in the chimeras are indicated on the left. The major domains of the proteins encoded by *gag* and *pol* are indicated as follows: MA, matrix; CA, capsid; NC, nucleocapsid; PR, protease; RT, reverse transcriptase; IN, integrase. (b) Replication of wild-type (WT) HIV-1_{NL4-3} and various chimeric viruses, as indicated, in Jurkat T cells. The accumulation of exogenous RT activity in the supernatant of infected cells (ordinate) is shown for the indicated day postinfection (abscissa).

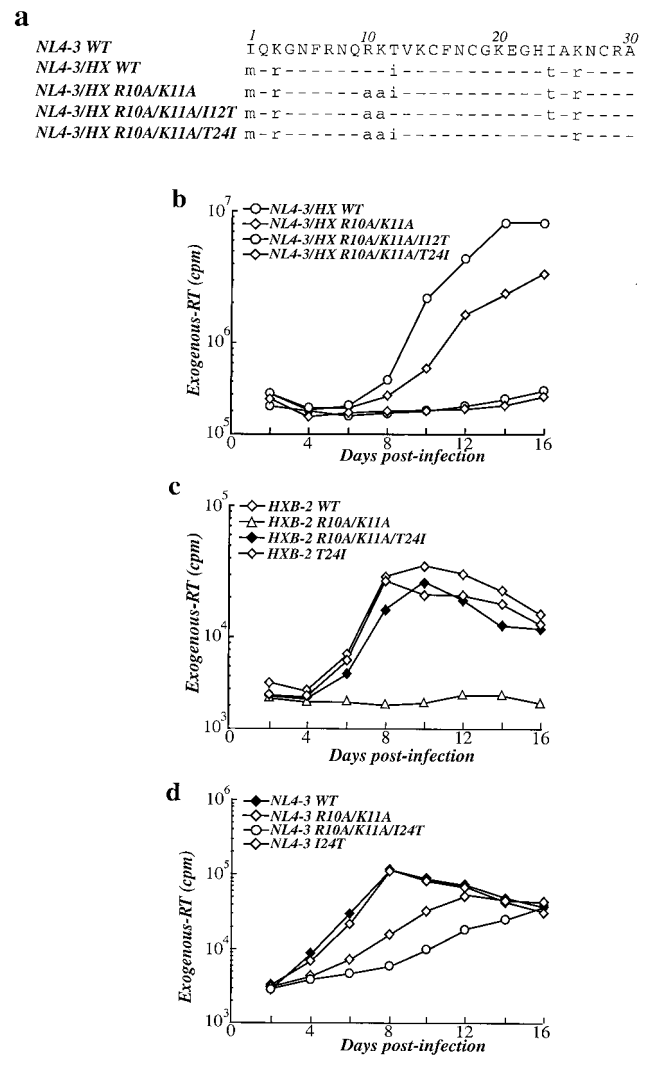


FIG. 3. Effect of NC residues that are not conserved between HIV-1_{NL4-3} and HIV-1_{HXB-2} on the phenotype of the R10A/K11A mutation. (a) Amino acid sequence alignment showing NC residues that are not conserved between HIV-1_{NL4-3} and HIV-1_{HXB-2}. Dashes indicate residues identical to HIV-1_{NL4-3}. Amino acid differences are indicated by lowercase letters (b to d). Replication kinetics following infection of Jurkat T cells with an HIV-$1_{NL4-3/HXB-2}$ chimera (b), complete HIV-1_{HXB-2} provirus (c), or complete HIV-1_{NL4-3} provirus (d) bearing the indicated mutations. The accumulation of exogenous RT activity in the supernatant of infected cells (ordinate) is shown for the indicated day postinfection (abscissa). WT, wild type.

three-fold less infectious than wild-type HIV-1_{NL4-3} virions (data not shown); again, the decreased infectivity of HIV-1_{HXB-2} might be explained by the nonfunctional Vpu, Vpr, and Nef in this virus (10, 11). For clarity, then, the infectivity of each mutant shown in Fig. 4a is presented as a percentage of the infectivity of the respective wild-type provirus.

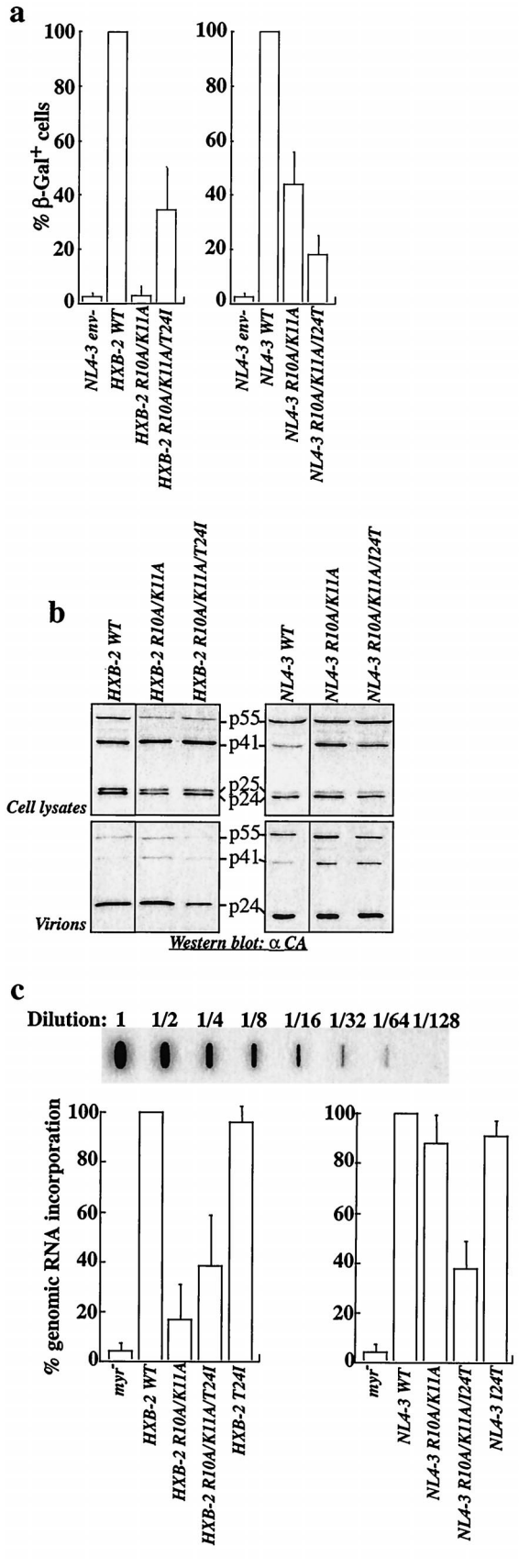
Virions containing the R10A/K11A mutation in the context of HIV-1_{HXB-2} were about 30-fold less infectious than wild-type HIV-1_{HXB-2} (Fig. 4a) or 90- to 100-fold less infectious than wild-type HIV-1_{NL4-3}. Changing T24 to the isoleucine found at this position in HIV-1_{NL4-3} increased the infectivity of HIV-1_{HXB-2} R10A/K11A virions more than 10-fold. In the context of HIV-1_{NL4-3}, the R10A/K11A mutation caused only a two- to threefold reduction in the number of β-galactosidase-positive cells. Changing I24 to the threonine found at this position in HIV-1_{HXB-2} decreased the infectivity of HIV-1_{NL4-3} R10A/K11A further (five- to six-fold lower infectivity than for wild-type HIV-1_{NL4-3}).

The effect of NC residue 24 on the biochemistry of virions harboring the R10A/K11A mutation was examined next. HIV-1_{HXB-2} and HIV-1_{NL4-3} proviral DNAs were transfected into 293T cells. Cell lysates were analyzed by Western blotting, as previously described (5), using an anti-CA antibody (Intracel, Cambridge, Mass.) that also recognizes p55 (the Gag polyprotein), p41 (a processing intermediate containing MA, CA, and the SP1 spacer peptide), and p25 (an intermediate containing CA and SP1). Although differences were observed between HIV-1_{HXB-2} and HIV-1_{NL4-3} in terms of the accumulation of Gag-processing intermediates (Fig. 4b), the accumulation of these products did not correlate with the presence of the R10A/K11A mutation or with the identity of NC residue 24. Western blot analysis of virions purified by ultracentrifugation through 25% sucrose did not reveal appreciable differences in the virion yield or in the degree of processing of Gag products at steady state (Fig. 4b). Similar results were obtained when Western blot analysis was carried out using anti-RT or anti-NC antibodies or after pulse-chase analysis followed by immunoprecipitation with serum from an HIV-1-infected individual (data not shown). These results suggest that the R10A/K11A mutation has no obvious effects on virion yield or on the stability and processing of viral proteins.

Finally, the amount of viral genomic RNA packaged into mutant virions was quantified using previously described methods (7). Virions produced as above were purified by ultracentrifugation through 25% sucrose, normalized by exogenous RT activity, and then transferred to a nylon membrane by using a dot-blot apparatus (Bio-Rad). RNA was detected by hybridization with a ^{32}P-end-labeled DNA oligonucleotide (5′-CGC GCCTTGGTTCTCTCATCTGGCCTGG-3′, antisense orientation, nucleotides 1459 to 1482) that hybridizes with a conserved portion of HIV-1 genomic RNA. Wild-type HIV-1_{HXB-2} virions were two- to threefold less efficient than wild-type HIV-1_{NL4-3} at incorporating viral genomic RNA. The difference in packaging efficiency between these two clones is unlikely to be due to differential annealing of the probe since the target RNA sequence is identical in the two clones.

Compared to wild-type HIV-1_{HXB-2}, the R10A/K11A mutation caused a fivefold decrease in viral genomic RNA packaging (Fig. 4c). In contrast, compared with wild-type HIV-1_{NL4-3}, no major effect on viral genomic RNA packaging was observed with the HIV-1_{NL4-3} R10A/K11A mutant. The decrease in RNA packaging of HIV-1_{HXB-2} R10A/K11A was corrected twofold by changing the threonine at residue 24 to isoleucine. RNA incorporation into HIV-1_{NL4-3} R10A/K11A virions was decreased approximately twofold when the isoleucine at residue 24 was changed to threonine. Introduction of changes at position 24 in the absence of other mutations did not affect viral genomic RNA incorporation. Thus, variations in RNA packaging correlated with, and possibly explain, the replication behavior of the different proviruses bearing the R10A/K11A mutation.

In conclusion, our results indicate that the context-depen-



dent replication phenotype of the R10A/K11A mutation depends mainly on the identity of the amino acid present at position 24 of NC. Our data suggest that effects of this residue on viral replication are exerted at the level of viral genomic RNA packaging. NC position 24 is a hydrophobic residue in almost all HIV-1 isolates (15); the hydrophilic threonine in HIV-1_{HXB-2} is a rare exception.

Solution structures have been determined for two *cis*-acting, HIV-1 packaging-signal stem-loops (SL3 and SL2) bound to HIV-1_{NL4-3} NC (1, 9). The isoleucine at HIV-1_{NL4-3} NC position 24 is part of a hydrophobic cleft that contacts the guanosine bases at position 9 of SL3 or position 11 of SL2. Based on these structural data and the observed effects on RNA packaging and viral replication of the mutations described here, it is reasonable to propose that, compared to HIV-1_{HXB-2} NC threonine 24, HIV-1_{NL4-3} NC isoleucine 24 confers tighter binding of the NC zinc finger domain to guanosine bases. Substitution of isoleucine by threonine would lead to weaker binding, reductions in packaging efficiency, and decreased infectivity. The NC basic residues R10 and K11 have electrostatic interactions with SL3 (9). If RNA binding by HIV-1_{HXB-2} NC were inherently weaker due to threonine 24, disruption of residues R10 and K11 by mutation would result in a noticeable replication phenotype only in the context of this provirus.

Amazingly similar to the findings presented here, a threonine-to-isoleucine change at position 24 of NC was previously reported as a second-site suppressor mutation that contributed to the rescue of replication and RNA packaging in an HIV-1_{HXB-2} derivative bearing a deletion in the RNA dimerization initiation site (16, 21). Residue 24 is the major determinant of the different R10A/K11A phenotypes reported here, but RT activity above the background accumulated in cultures infected by HIV-1_{NL4-3} R10A/K11A/I24T, indicating that residue 24 differences are not sufficient to explain the different phenotypes with the two proviruses. Less significant contributions

FIG. 4. Characterization of HIV-1_{HXB-2} and HIV-1_{NL4-3} virions bearing NC mutations. 293T cells were transfected with the indicated proviral DNAs. Virions were purified from culture supernatant by ultracentrifugation through 25% sucrose and then normalized by exogenous RT. (a) Infectivity of the virion preparations was determined by counting β-galactosidase-positive (β-gal$^+$) cells 2 days after infection of CD4$^+$ HeLa reporter cells (MAGI assay). Data for each mutant are presented as the percentage with respect to the respective wild-type (WT) virus. The bar graph shows results obtained from six independent experiments with standard errors of the mean. NL4-3 env- indicates a virus lacking a functional *env*. (b) Western blot probed with anti-CA antibody. 293T cell lysates are shown in the upper panels. Virion-associated proteins are shown in the lower panels. The positions of migration of the various *gag* products recognized by this antibody are indicated. (c) Virion-associated RNA prepared from normalized amounts of virions was loaded onto a nylon membrane and probed with a ^{32}P-end-labeled DNA oligonucleotide specific for viral genomic RNA. The signal obtained after hybridization was quantified with a phosphorimager. A representative standard curve obtained after dilution of genomic RNA is shown here and illustrates the linearity of the method used. Results are presented as percentage with respect to the wild-type viruses. The bar graph presents results obtained from two to four independent experiments with standard errors of the mean. myr$^-$ indicates a control preparation from cells transfected with HIV-1_{NL4-3} bearing the Gag G1A mutation that disrupts Gag myristylation. This preparation does not contain virions and is used here to monitor for DNA contaminants derived from transfection.

might be made by other differences in coding sequences or perhaps in 5′ leader sequences.

We thank Cagan Gurer and Michael Summers for critical reading of the manuscript.

This work was supported by grant AI 41857 (J.L.) and by shared core facilities of the Columbia-Rockefeller Center for AIDS Research (P30 AI42848), both from the National Institutes of Health.

REFERENCES

1. **Amarasinghe, G. K., R. N. De Guzman, R. B. Turner, K. J. Chancellor, Z. R. Wu, and M. F. Summers.** 2000. NMR structure of the HIV-1 nucleocapsid protein bound to stem-loop SL2 of the psi-RNA packaging signal. Implications for genome recognition. J. Mol. Biol. **301:**491–511.
2. **Berkowitz, R., J. Fisher, and S. P. Goff.** 1996. RNA packaging. Curr. Top. Microbiol. Immunol. **214:**177–218.
3. **Carteau, S., S. C. Batson, L. Poljak, J. F. Mouscadet, H. de Rocquigny, J. L. Darlix, B. P. Roques, E. Kas, and C. Auclair.** 1997. Human immunodeficiency virus type 1 nucleocapsid protein specifically stimulates Mg^{2+}-dependent DNA integration in vitro. J. Virol. **71:**6225–6229.
4. **Carteau, S., R. J. Gorelick, and F. D. Bushman.** 1999. Coupled integration of human immunodeficiency virus type 1 cDNA ends by purified integrase in vitro: stimulation by the viral nucleocapsid protein. J. Virol. **73:**6670–6679.
5. **Cimarelli, A., and J. Luban.** 1999. Translation elongation factor 1-alpha interacts specifically with the human immunodeficiency virus type 1 Gag polyprotein. J. Virol. **73:**5388–5401.
6. **Cimarelli, A., and J. Luban.** 2000. Human immunodeficiency virus type 1 virion density is not determined by nucleocapsid basic residues. J. Virol. **74:**6734–6740.
7. **Cimarelli, A., S. Sandin, S. Hoglund, and J. Luban.** 2000. Basic residues in human immunodeficiency virus type 1 nucleocapsid promote virion assembly via interaction with RNA. J. Virol. **74:**3046–3057.
8. **Cimarelli, A., S. Sandin, S. Hoglund, and J. Luban.** 2000. Rescue of multiple viral functions by a second-site suppressor of a human immunodeficiency virus type 1 nucleocapsid mutation. J. Virol. **74:**4273–4283.
9. **De Guzman, R. N., Z. R. Wu, C. C. Stalling, L. Pappalardo, P. N. Borer, and M. F. Summers.** 1998. Structure of the HIV-1 nucleocapsid protein bound to the SL3 psi-RNA recognition element. Science **279:**384–388.
10. **Emerman, M., and M. H. Malim.** 1998. HIV-1 regulatory/accessory genes: keys to unraveling viral and host cell biology. Science **280:**1880–1884.
11. **Fisher, A. G., E. Collalti, L. Ratner, R. C. Gallo, and F. Wong-Staal.** 1985. A molecular clone of HTLV-III with biological activity. Nature **316:**262–265.
12. **Gorelick, R. J., W. Fu, T. D. Gagliardi, W. J. Bosche, A. Rein, L. E. Henderson, and L. O. Arthur.** 1999. Characterization of the block in replication of nucleocapsid protein zinc finger mutants from moloney murine leukemia virus. J. Virol. **73:**8185–8195.
13. **Kimpton, J., and M. Emerman.** 1992. Detection of replication-competent and pseudotyped human immunodeficiency virus with a sensitive cell line on the basis of activation of an integrated beta-galactosidase gene. J. Virol. **66:**2232–2239.
14. **Kodera, Y., K. Sato, T. Tsukahara, H. Komatsu, T. Maeda and T. Kohno.** 1998. High-resolution solution NMR structure of the minimal active domain of the human immunodeficiency virus type-2 nucleocapsid protein. Biochemistry **37:**17704–17713.
15. **Kuiken, C., B. Foley, B. Hahn, P. Marx, F. McCutchan, J. W. Mellors, J. Mullins, S. Wolinsky, and B. Korber.** 1999. Human retroviruses and AIDS 1999: a compilation and analysis of nucleic acid and amino acid sequences. Los Alamos National Laboratory, Los Alamos, N.M.
16. **Liang, C., L. Rong, M. Laughrea, L. Kleiman, and M. A. Wainberg.** 1998. Compensatory point mutations in the human immunodeficiency virus type 1 Gag region that are distal from deletion mutations in the dimerization initiation site can restore viral replication. J. Virol. **72:**6629–6636.
17. **Poon, D. T., J. Wu, and A. Aldovini.** 1996. Charged amino acid residues of human immunodeficiency virus type 1 nucleocapsid p7 protein involved in RNA packaging and infectivity. J. Virol. **70:**6607–6616.
18. **Rein, A.** 1994. Retroviral RNA packaging: a review. Arch. Virol. Suppl. **9:** 513–522.
19. **Rein, A., L. E. Henderson, and J. G. Levin.** 1998. Nucleic-acid-chaperone activity of retroviral nucleocapsid proteins: significance for viral replication. Trends Biochem. Sci. **23:**297–301.
20. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
21. **Shen, N., L. Jette, C. Liang, M. A. Wainberg, and M. Laughrea.** 2000. Impact of human immunodeficiency virus type 1 RNA dimerization on viral infectivity and of stem-loop B on RNA dimerization and reverse transcription and dissociation of dimerization from packaging. J. Virol. **74:**5729–5735.
22. **Weiss, A., R. Wiskocil, and J. Stobo.** 1984. The role of T3 surface molecules in the activation of human T cells: a two stimulus requirement for IL-2 production reflects events occurring at a pretranslational level. J. Immunol. **133:**123–128.